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TAp73 upregulates IL-1β in cancer cells: Potential biomarker in lung and breast cancer?

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ABSTRACT

p73 is a transcription factor belonging to the p53 tumour suppressor family. $p73^{-/-}$ mice exhibit a range of phenotypes including neurological, reproductive and inflammatory defects. Although the role of p73 in the control of genomic stability explains part of these phenotypes, a clear mechanism of how p73 participates in the inflammatory response is still elusive. Interleukin-1β (IL-1β) has a crucial role in mediating the inflammatory response. Because of its high potency to induce inflammation, the activation and secretion of IL-1β is tightly regulated by large protein complexes, named inflammasomes. Inflammasomes regulate activation of proinflammatory caspase-1, which in turn proteolytically processes its substrates, including pro-IL-1β. Caspase-1 gene transcription is strongly activated by p53 protein family members including p73. Here, we have addressed whether p73 might be directly involved in IL-1β regulation and therefore in the control of the inflammatory response. Our results show that TAp73β upregulates pro-IL-1β mRNA and processed IL-1β protein. In addition, analysis of breast and lung cancer patient cohorts demonstrated that interaction between p73 and IL-1β predicts a negative survival outcome in these human cancers.

1. Introduction

p73 belongs to the p53 family of transcription factors (p53, p63 and p73), that share a high degree of structural homology, especially in their DNA-binding domains [1]. The *TP73* gene generates multiple protein isoforms, which arise as a result of alternative promoter usage and differential mRNA splicing at the 3'end. The use of the second promoter generates ΔNp73 isoforms that lack the transactivation (TA) domain present in the N terminus of the full length p73 protein (TAp73), and alternative splicing gives rise to 7 isoforms (α − η) with different C-terminal sequences that vary in specificity and activity [2,3]. The p53 family is considered among the most powerful family of genes, having a significant impact on several biological processes [4,5] ranging from cell cycle regulation [6]/apoptosis [7,8], genome stability [8] and metabolism [9–11] to organ development/homeostasis [12,13] and fertility [4,14]. p73 itself has been implicated in many of these processes, including the control of genome stability which is associated with its capacity to exert tumour suppression and act as a reproduction censor [4,15–18]. However, the original p73-deficient mouse, lacking all p73 isoforms, shows no increased susceptibility to spontaneous tumours, but a rather diffuse inflammation and infection, associated with severe rhinitis and purulent otitis media with massive neutrophil infiltration. Despite these indications of inflammation and infection, no obvious deficiencies in lymphoid or granulocyte populations were detected in $p73^{-/-}$ mice [1]. A recent analysis of p73 null phenotypes identified a potential unifying mechanism for these diverse phenotypes [19,20]. Marshall et al. showed that p73 is required for the formation of multiciliated epithelia, and p73 binds in close proximity to more than 100 cilia-associated genes that are required for the development and maintenance of multiciliated cells. Loss of ciliary biogenesis provides a unifying mechanism for many phenotypes observed in $p73^{-/-}$ including hydrocephalus; hippocampal dysgenesis; sterility; and chronic inflammation/infection of the lung, middle ear, and sinus [19,20].

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Unlike the full p73$^{-/-}$ mice, TAp73$^{-/-}$ mice show increased susceptibility to spontaneous and carcinogen-induced tumours [16,21]. They also display higher circulating levels of proinflammatory cytokines in response to LPS treatment suggesting that TAp73 has a role in macrophage polarization and innate immunity [22]. These data suggest the possibility of a causative correlation between the cancer phenotype and chronic inflammation for the establishment of a tumorigenic context [22]. A function for p73 in regulating inflammation is also suggested by several reports that link p73 expression to inflammatory diseases such as gastritis and otitis media [23,24]. However the mechanism and the molecular basis behind these inflammatory effects remain largely unclear.

Inflammasomes are multiprotein complexes containing a member of the NOD-like receptor (NLR) family, such as NLRP3 and IPAF that act as important effectors of the innate immune system. The NLR protein recruits the inflammasome-adaptor protein ASC, which in turn interacts with caspase-1 leading to its activation. Once activated, caspase-1, also known as interleukin-1β converting enzyme, promotes the maturation of the proinflammatory cytokines interleukin (IL)-1β and IL-18 and cause pyroptosis, a type of inflammatory cell death [6,25–27]. However, unlike IL-18 which is constitutively expressed, activation of IL-1β requires concomitant transcriptional activation of the unprocessed IL-1β (pro IL-1β) gene; this is mainly mediated by nuclear factor-κβ (NF-κβ) [28]. Processed IL-1β, generated upon caspase-1 activation, provides positive feed-forward stimulation for inflammatory cytokines, thereby amplifying inflammation. Inflammasomes have been linked to a variety of autoinflammatory and autoimmune diseases, including neurodegenerative disease, metabolic disorders and cancer [27,29].

Previous reports have described human *caspase-1* as a direct target of all p53-family proteins [30–32]. It has been shown that p73α and p73β isoforms exert a substantial positive transcriptional activation on the *Caspase-1* promoter, leading to a significant upregulation of caspase-1 expression upon TAp73 activation. Based on this and older evidence we wanted to investigate whether p73 plays a direct role in the regulation of IL-1β. This regulation might reflect an influence of TAp73 on inflammasomes and might in turn have important implications for tumour inflammation.

2. Materials and methods

2.1. Cell culture

The human non-small cell lung carcinoma cell line NCI-H1299 was maintained at 37 °C in a CO_2 incubator in RPMI medium, containing L-glutamine, 4.5 g/L D-Glucose, 2.383 g/L HEPES Buffer, 1.5 g/L Sodium Bicarbonate, 110 mg/L Sodium Pyruvate (Gibco, Life Technologies), supplemented with 10% FBS (Labtech) and with Penicillin/Streptomycin (Gibco, Life Technologies).

2.2. Cell transfection

For overexpression, H1299 cells were seeded 24 h before transfection. Transfection was performed with 10 μg DNA (pcDNA empty, pcDNA HA-TAp73α, pcDNA HA-TAp73β, pcDNA ΔN-TAp73α, pcDNA HA-p53 wt, pcDNA HA-p53R273H) per 10 cm dish with 1.2×10^6 cells seeded using Lipofectamine 2000 Reagent (Invitrogen). Cells were collected 24 h after transfection.

For TAp73 knockdown in H1299 cells, 1.2×10^6 cells were seeded per 10 cm dish 24 h before transfection. Transfection was performed using 50 nM siRNA (control siRNA or siTAp73-1 and siTAp73-2 (Ambion)) and Lipofectamine RNAiMAX (Invitrogen). Each dish was split in two 24 h after transfection and cells were collected 48 h after transfection.

2.3. RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells using the RNEasy Mini Kit (Qiagen), according to the Qiagen company protocol. 2 μg of total RNA was used to prepare cDNA using RevertAid H minus First strand cDNA Synthesis kit (ThermoScientific), using Random primers and the protocol from the kit. qPCR was performed using 1/10 of the prepared cDNA and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was analyzed in accordance with 7500 Software version 2.0.6 of Applied Biosystems. Gene expression levels were quantified according to the comparative ΔΔCt method and normalized to expression of the TBP housekeeping gene. Sequences of the primers used for the qPCR are: human TAp73 Fw CAGACAGCACCTACTTCGACCTT, Rev CCGCCCACCACCTCATTA; p21 Fw CCTGTCACTGTCTTGTACCCT, Rev GCGTTTGGAGTGGTAGAAATCT; p53 Fw CCCAAGCAATGGATGATTTGA, Rev GGCATTCTGGGAGCTTCATCT; DNp73; Caspase-1 Fw TGCCTGTTCCTGTGATGTGG, Rev TGTCCTGGGAAGAGGTAGAAACATC; human IL-1β Fw TGCACCTGTACGATCACTGAAC, Rev TTGCTCCATATCCTGTCCCTG; TBP Fw TCAAACCCAGAATTGTTCTCCTTAT, Rev CCTGAATCCCTTTAGAATAGGGTAGA.

2.4. Western blot analysis

For protein extraction, cells were lysed in RIPA buffer with protease inhibitor cocktail tablets Complete, EDTA-free (Roche) and phosphatase inhibitor cocktail tablets PhosSTOP (Roche). Lysates were measured for protein concentration by using the Bio-Rad Protein Assay (Bio-Rad), then mixed with Laemmli loading buffer, electrophoresed on SDS-PAGE gels and separated proteins transferred to PVDF blotting membranes (Amersham, GE Healthcare). Membranes were blocked for 1 h in 10% (m/vol) dry milk dissolved in TBS with 1% (vol/vol) Tween-20 (TBSt); incubated with primary antibodies overnight and with secondary antibodies conjugated with horseradish peroxidase, for 1 h. Antibodies were diluted in 10% dry milk in TBSt: anti-HA 1:1000 (Covance), anti-GAPDH 1:40000 (Sigma), anti-p21 1:1000 (Santa Cruz Biotechnology), anti-p73 1:3000 (Bethyl). To detect the signal ECL Western Blotting Detection Reagent (Amersham, GE Healthcare) or SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) was used.

2.5. IL-1β enzyme-linked immunosorbent assay (ELISA)

P73 was overexpressed in H1299 cells as described above, and cells were lysed 24 h after transfection in Non-denaturing lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% NP-40). Supernatants were collected and concentrated 20 times using Amicon Ultra-2mL Centrifugal filters Ultracel-10K (Merk Milipore). Supernatants and cell lysates were collected and the level of IL-1β was quantified using the Human IL-1β/IL-1F2 Quantikine ELISA kit (R&D Systems) following the manufacturer's instructions. Quantification was performed by measuring the absorbance at 450 nm with 540 as a reference using the Infinite M200 PRO (TECAN) microplate reader.

2.6. Chromatin immunoprecipitation assay

TAp73β was overexpressed for 24 h. Cells were collected at 80% confluency and fixed in 37% formaldehyde and subjected to sonication for DNA shearing. Chromatin was sonicated and immunoprecipitated with/without 10 μL anti-HA antibodies (Covance) or 10 μL nonspecific immunoglobulin G (IgG) antibodies (Invitrogen) using the MAGnify ChIP System kit (Invitrogen). The co-immunoprecipitated DNA fragments were amplified by qPCR. P21 and MDM2 were used as positive controls; L32 was used as negative control. The primers used were: IL-1β (RE in Exon1) Fw

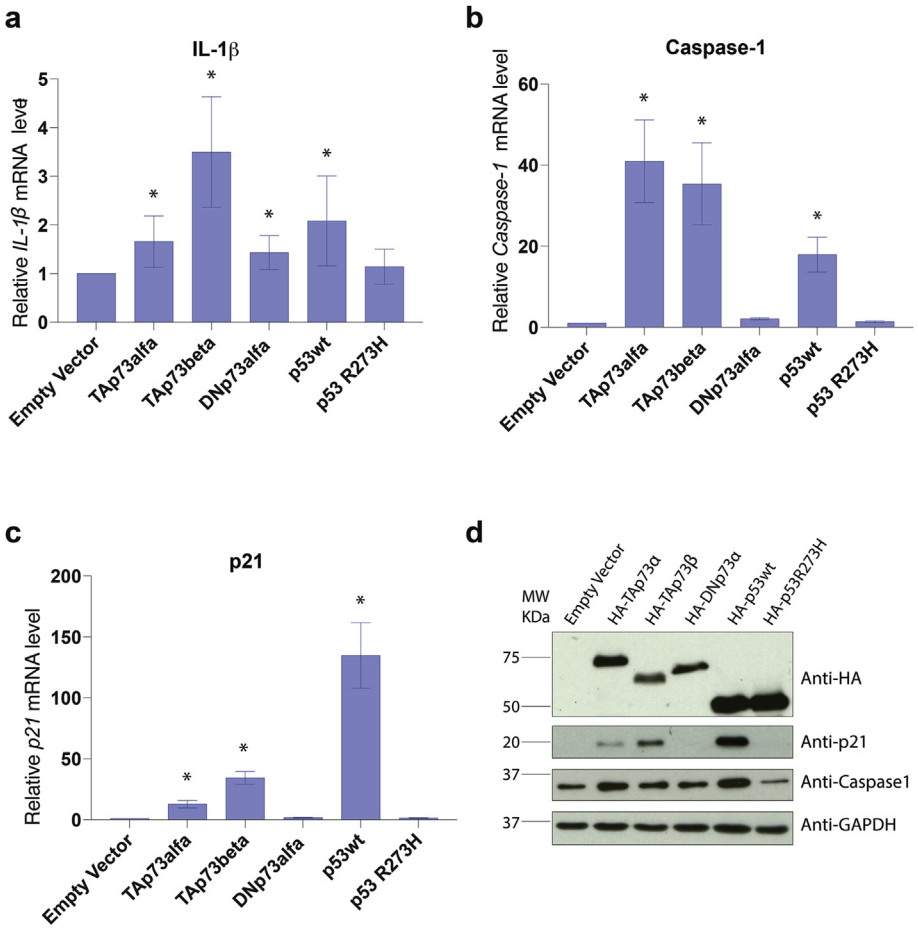

Fig. 1. TAp73 overexpression increases expression of *IL-1β* in a non-small cell lung cancer cell line. HA-tagged p73 isoforms (TAp73α, TAp73β and ΔNp73) and HA-tagged p53 wt and mutant p53R273H were overexpressed in the H1299 cell line for 24 h (**A**) mRNA level of *IL-1β*; (**B**) mRNA level of *Caspase-1*; (**C**) mRNA level of *p21* were analyzed by qPCR. Relative expression of genes was normalized against TBP and calculated as fold induction. The data shown are mean ± S.D. (n = 4) *P < 0.05 (Student's T-test). (**D**) Protein levels of HATAp73, p21, Caspase-1 and GAPDH were analyzed by WB. Figures show a representative replicate of 3 independent experiments.

ACTGGCAGATACCAAACCTCTTCG, Rev TACACACAAAGAGGCAGAGAGACAG; IL-1β (RE in Intron5) Fw TCCTGGCAGCTTGCTAATTCTCC, Rev GACTTGGGTGGACATGGTCCTG; MDM2 Fw GGTTGACTCAGCTTTTCCTCTTG, Rev GGAAAATGCATGGTTTAAATAGCC; p21 Fw GGCTGGTGGCTATTTTGTCC, Rev CCCCTTCCTCACCTGAAAACA; L32 Fw TTCCTGGTCCACAACGTCAAG, Rev TGTGAGCGATCTCGGCAC.

2.7. Bioinformatic analyses

Bioinformatic analysis was performed as previously described [11]. Different cancer patient datasets (GSE31120; GSE3494; GSE17537; GSE25136; and Van't Verr et al.) were used for the analysis. The survival estimation analysis in p73/IL-1β positive and negative groups was performed computing the correlation between p73 and IL-1β expression profiles. The samples were split in two cohorts: “gene correlation” or “no correlation” in order to group all samples with a maximal effect on correlation (maximal increase in positive correlation) in the first cohort. The computation was repeated until there would be no sample which could be removed from cohort 1 to cohort 2 to increase the positive correlation between p73 and IL-1β. The separation of patients into “cohort 1” and “cohort 2” along with survival information was then used to compute survival outcome. The R statistical package was used to perform survival analyses and to draw KMplots.

2.8. Statistics

Technical as well as biological triplicates of each experiment were performed. Error bars indicate ±S.D. in each figure. Statistical significance was determined using the unpaired two-tailed Student's *t*-test using GraphPad Software. A p-value ≤0.05 was considered statistically significant.

3. Results

3.1. TAp73 induces expression of IL-1β

To evaluate the ability of different isoforms of p73 to regulate the expression of interleukin-1β (IL-1β), we used the p53-null human non-small cell lung carcinoma cell line NCI-H1299, expressing endogenous TAp73. First, we overexpressed HA-tagged p73 isoforms (TAp73α, TAp73β and ΔNp73) and HA-tagged p53 wt and the p53R273H mutant for 24 h. To evaluate whether p73 activation was associated with induced transcription of the *IL-1β* gene, we performed real-time quantitative PCR. qPCR showed a 3.5 fold increase of *IL-1β* mRNA level after TAp73β overexpression, a 2 fold increase after p53 overexpression and a 1.5 fold increase after overexpression of TAp73α and ΔNp73 (Fig. 1a). Protein and RNA levels of p73 isoforms (TAp73α, TAp73β and ΔNp73) and HA-tagged p53 wt and p53R273H mutant were

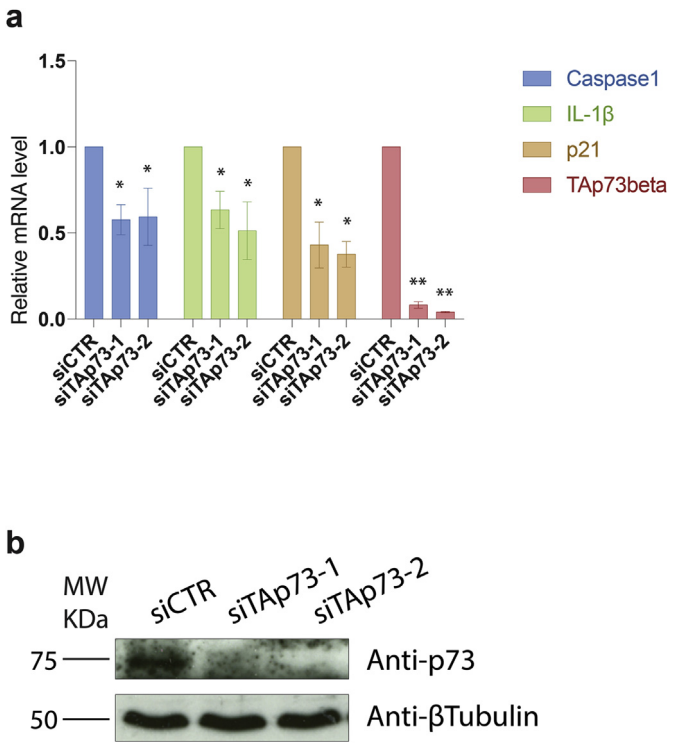

Fig. 2. TAp73 regulates expression of *IL-1β*. (**A**) mRNA levels of *IL-1β*, *Caspase-1*, *p21* and TAp73 were analyzed by qPCR after TAp73 knockdown in the H1299 cell line for 48 h. Relative expression of the genes was normalized against *TBP* and calculated as fold induction. The data shown are mean ± S.D. (n = 4). *P < 0.05 (Student's T-test). (**B**) The protein level of p73 after 48 h of TAp73 knockdown was analyzed by WB. Figures show a representative replicate of 3 independent experiments.

strongly upregulated (Fig. 1d, Supplementary Figs. S1a–c). Upregulation of transcriptional targets *p21* and *Caspase-1* confirmed p73 and p53 transcriptional activation (Fig. 1b, c, d).

To provide further evidence for p73-induced upregulation of *IL-1β* we performed knockdown experiments in H1299 cells by transfecting selective siRNAs for TAp73 isoforms. mRNA levels of *IL-1β* were downregulated after TAp73 silencing, as were the levels of *Caspase-1* and *p21* mRNA (Fig. 2a). Western blot analysis confirmed TAp73 depletion (Fig. 2b).

Collectively these data demonstrate TAp73-dependent upregulation of *IL-1β* expression at the RNA level both after TAp73 overexpression and after TAp73 knockdown.

3.2. *IL-1β is not a direct target of TAp73*

To investigate the possibility that TAp73 is able to directly bind a responsive element (RE) in the promoter region, we performed bioinformatics analysis to identify putative consensus p53RE elements in the promoter sequence of human IL-1β. We used MatInspector Professional software, which allows identification of p53-like REs. Within the first 1300bp upstream and 1300bp downstream of transcription start site we identified one putative binding site, which was located in Exon1 of the human IL-1β gene between +13 and +38bp (Fig. 3a). Another putative RE for p53 family proteins was found trough the p53FamTaG database [33]. This RE was located in Intron5 of human IL-1β (Fig. 3b). To validate the hypothesis of the direct regulation of IL-1β gene by p73 we performed chromatin immunoprecipitation (ChIP) assays for the two putative p53REs on the *IL-1β* gene after overexpression of the TAp73β isoform. The results show that p73 does not bind to either consensus RE (Fig. 3c,d). For positive controls, p21 and MDM2 promoters primers were used, with L32 primers as negative control (Fig. 3e).

These results suggest that p73 promotes *IL-1β* expression not by the direct interaction with the indicated REs of the *IL-1β* gene but by another indirect mechanism.

3.3. *TAp73β upregulates the mature IL-1β level*

TAp73 strongly upregulates caspase-1. Hence, we reasoned that TAp73 could regulate pro-IL1β processing rather than by direct transactivation of the gene, thus increasing the level of mature IL-1β. In order to investigate this, we performed the enzyme-linked immunosorbent assay for the presence of IL-1β following overexpression of p73 isoforms (TAp73α, TAp73β and ΔNp73) and HA-tagged p53 wt and the p53R273H mutant for 24 h. ELISA assay performed on the cell lysates showed increases in the level of IL-1β in the sample after overexpression of the TAp73β isoform, with a 10 pg/mL concentration of IL-1β in this sample (Fig. 4a). However, no detectable levels of IL-1β were observed in the supernatants, even after 20 fold concentration, possibly indicating the inability of the H1299 cells to secrete IL-1β. Therefore this result shows that TAp73β can affect the maturation of IL-1β, but not the mechanism of IL-1β secretion.

3.4. *Correlation of TAp73 and IL-1β expression level predicts poor patient prognosis*

Cancer inflammation has been associated with poor patient prognosis and therapy resistance. Consistently, high levels of IL-1β have been shown to represent a negative prognostic marker. In order to understand better the clinical relevance of p73-dependent regulation of IL-1β, we evaluated the biological consequence of the correlation between p73 and IL-1β. We used selected gene expression datasets of human lung and breast cancers annotated with patient survival information. Each group was split in two different groups: the first included all the samples to maximize a positive correlation between p73 and IL-1β, while in the second cohort all the other samples were included. Thus, we clustered the datasets into a group where the p73/IL-1β axis was present (gene interaction) and a second in which it was absent (NO interaction). The cohort with 'gene interaction' showed a significantly worse survival outcome compared to the cohort 'NO interaction' (Fig. 4b,c). Thus, bioinformatics data provide evidence of poorer survival in cancer patients with a positive correlation between p73 and IL-1β. Hence, these data highlight a potential role for a TAp73-dependent regulation of the inflammasome, thus highlighting a role for this axis in cancer pathogenesis.

4. Discussion

Our data demonstrate a relationship between TAp73 and IL-1β, although this is not occurring through direct transcriptional control, suggesting that there might be additional participants in this functional axis. TNFR/NF-κβ and IL1R/NF-κβ are major players involved in transcriptional induction of pro-IL-1β. Under inflammatory conditions, activation of these cytokine-mediated pathways promotes stabilization, nuclear translocation and activation of NF-κβ. Concomitantly, cytoplasmic caspase-1 is ready to promote processing of pro-IL-1β to IL-1β. Upon TAp73 overexpression we observed increased mRNA levels of pro-IL-1β as well as increased levels of mature IL-1β. Thus, TAp73 exerts a dual action on IL-1β activation (Fig. 4d). While the TAp73-dependent processing of pro-IL-1β can be ascribed to direct transcriptional regulation of caspase-1, the mechanism underlining the accumulation of pro-IL-1β mRNA

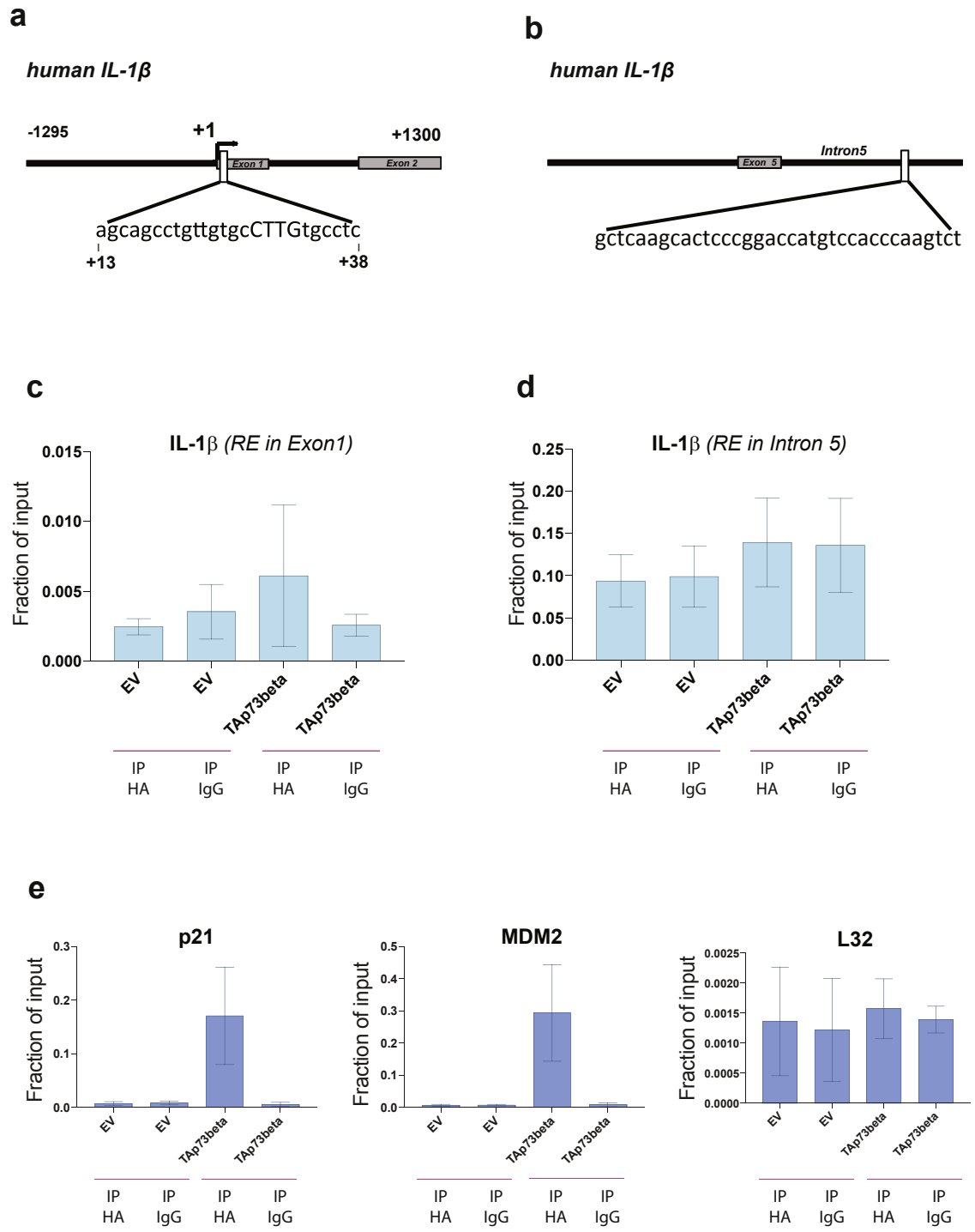

Fig. 3. Chromatin immunoprecipitation assay. (**A**) Schematic image of IL-1β RE located in Exon1. (**B**) Schematic image of IL-1β RE located in Intron5. (**C and D**) Chromatin extracted from H1299 with overexpressed HA-TAp73β was incubated with anti-HA or IgG antibodies. (**C**) Immunoprecipitated DNA was tested by qPCR for p53-Response element in Exon 1 of IL-1β gene. (**D**) Immunoprecipitated DNA was tested by qPCR for p53-Response element in Intron 5 of IL-1β gene. (**E**) ChIP on p21 and MDM2 promoter was performed as positive control and L32 was used as negative controls. Mouse IgG antibody was used as a negative control of the ChIP procedure. The data shown are mean ± S.D. (n = 3).

remains elusive. TNFR/NF-κβ pathway can regulate stability of TAp73 [34], however it is unknown whether TAp73 might influence NF-κβ in the transcriptional regulation of pro-IL-1β. More studies are needed to reveal the molecular mechanisms underlining our observation.

Unlike most cytokines, IL-1β does not have a signal peptide and therefore it is not secreted through the classical secretory pathway [35]. Secretion of IL-1β is a multistep process that requires both upregulation of the precursor pro-IL-1β (31kD) and processing of pro-IL-1β by caspase-1 to form its bioactive mature form (17kD). Without this step, pro-IL-1β is polyubiquitinated and degraded by the proteasome [36]. There are data showing that there is a fraction of cellular IL-1β localised to the vesicles of an endolysosomal nature that is targeted for degradation, but that can be redirected to the

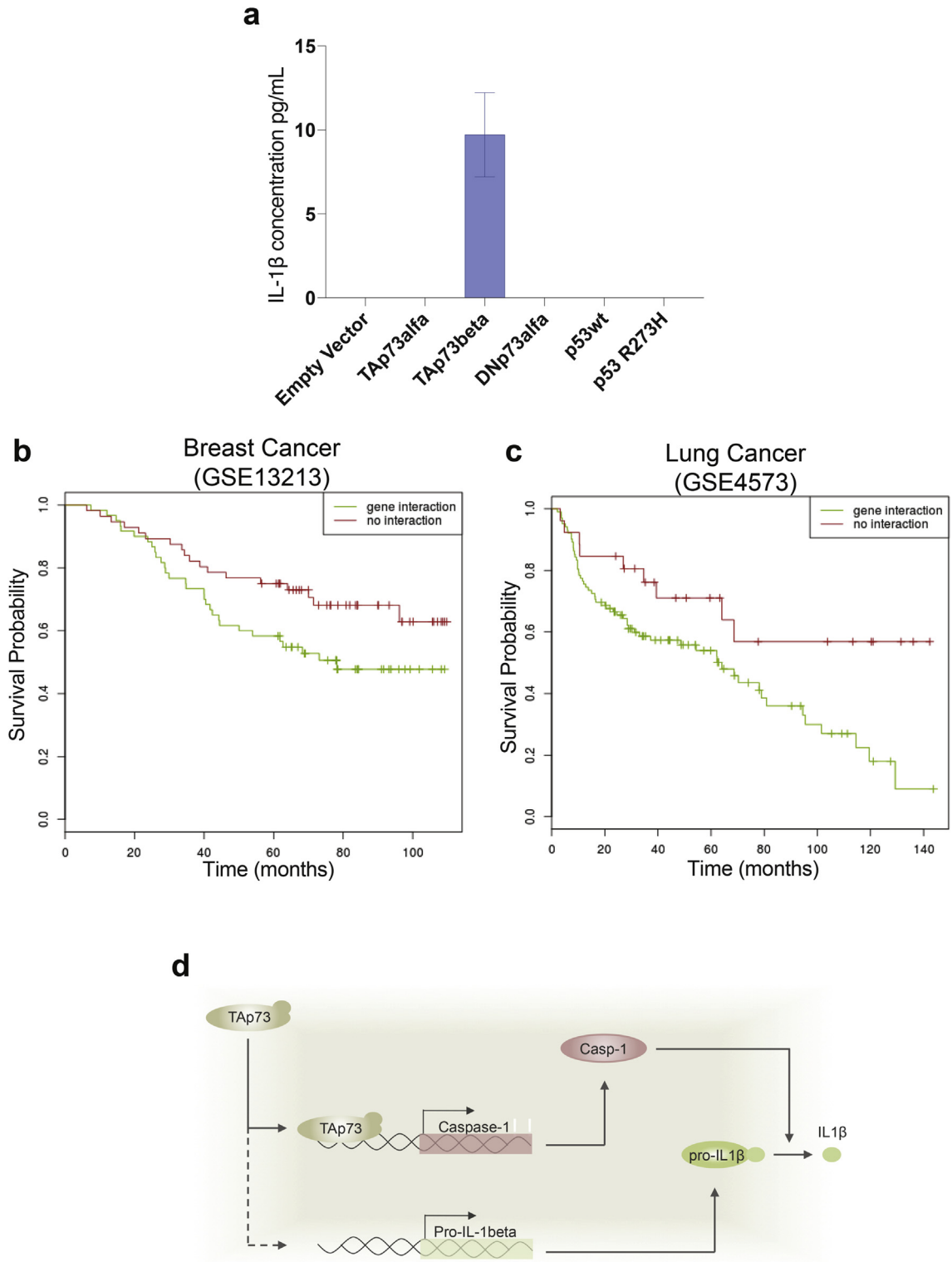

Fig. 4. (**A**) TAp73β upregulates protein level of IL-1β in the H1299 cell lysates. HA-tagged p73 isoforms (TAp73α, TAp73β and ΔNp73) and HA-tagged p53 wt and p53R273H mutant were overexpressed in H1299 cells for 24 h. Lysates were then harvested and analyzed for the presence of IL-1β using a cytokine-specific ELISA. The diagram displays the IL-1β content (pg/mL) of cell lysates (intracellular IL-1β). The data shown are mean ± S.D. (n = 3). (**B and C**) Panels represent patient survival estimation datasets of p73/IL-1β −positive correlation groups compared with negative or absent correlation groups Patients with (**B**) breast cancer and (**C**) lung cancer. Patients were divided in two groups: group one − gene interaction (positive correlation) and group two − no interaction (negative or absent correlation). (**D**) Schematic representation of the dual regulation exerted by TAp73 on IL-1β. TAp73 overexpression promotes upregulation of IL-1β mRNA, however the transcriptional control does not appear to be mediated by a direct binding of TAp73 on IL-1β gene locus. Additionally, TAp73 directly promotes expression of caspase-1, thus promoting processing of pro-IL-1β to IL-1β.

extracellular space following an appropriate secretory stimulus. However, the exact mechanism of secretion is poorly understood. Many observations probably exclude a single mechanism of secretion, but suggest that there are multiple mechanisms [37].

Reports indicate that ELISA kits calibrated using mature IL-1β as a standard will detect, but considerably underestimate, the unprocessed IL-1β precursor form present in the sample [38,39]. The complexity of the secretion process might explain the intracellular accumulation of IL-1β we observed in our lung cancer cell line model. Additional stimuli might be required in H1299 to secrete matured IL-1β. The use of an in vitro cell line indeed does not appropriately recapitulate the conditions in which lung cancer cells might produce and secrete IL-1β in the tumour microenvironment. In fact, IL-1β is produced by many cell types, although the majority of studies focus on its production by cells of the innate immune system, such as monocytes and macrophages. The present study raises the question about the regulation of IL-1β in non-immune cells, although it still leaves open the possibility that TAp73 triggers the upregulation and secretion of IL-1β in monocyte-macrophage cell lines, such as the human THP.1 cells.

p73 plays a role in various cellular signalling pathways during development and growth control, and the TA isoform has tumour suppressor properties. Several isoforms of p73 exist with considerable differences in their function. Whereas the functions of the N-terminal isoforms (TA and ΔNp73) and their opposing pro- and antiapoptotic roles have become evident, the functional differences of the distinct C-terminal splice forms of TAp73 are not so clear. Interestingly, our data show that overexpression of TAp73β isoform leads to greater upregulation of *IL-1β* mRNA, and is the only isoform that upregulates intracellular level of IL-1β.

IL-1β has found to be elevated in various types of cancers, and it is known that IL-1β producing tumours have a bad prognosis [40,41]. Our data are in agreement with these observations and expand the prognostic value to the correlation between TAp73 and IL-1β. This suggests that IL-1β could be a potential biomarker of cancer prognosis. Because IL-1β can have both beneficial and harmful effects, it especially important to know how this cytokine is activated. There are contradicting data showing on one hand that excessive inflammasome activation could possibly drive tumorigenesis and on the other hand data that shows that the loss of inflammasome activation could also increase cancer progression [42–44]. *Caspase-1* mRNA is strongly upregulated and it is known to be controlled by p73 directly. More studies need to be done to determine the mechanism of p73 influence on inflammasomes. This report provides evidence that p73 and particularly the TAp73β isoform affects IL-1β at both mRNA and maturation. Together with the previous data that shows the direct regulation of caspase-1 by p73 we hypothesise that there is a regulation of inflammasomes by p73.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.10.085.

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